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CXCL12 Derived From ACKR1+ Intraplaque Neovessels Mediates CD8+ T Cell Recruitment in Human Atherosclerosis

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Atherosclerosis is the most common underlying cause of life-threatening cardiovascular events. In contrast with the more traditional view of macrophages and foam cells dominating the atherosclerotic landscape, recent advances in immunophenotyping have revealed that T cells—in particular CD8+ T cells—are the most abundant hematopoietic population in human atherosclerotic plaques.[1] To better understand the role that T cells play in the development and progression of atherosclerosis, as well as their impact on clinical outcomes, it is crucial to identify the pathways that regulate their recruitment to atherosclerotic plaques.

Building on the previous finding that biopsies from carotid artery disease patients could be kept in culture for several days,[2] we developed a 3-dimensional (3D) tissue-culture model to study leukocyte recruitment to human atherosclerotic plaques. The study was approved by an institutional review committee (No. 2799/10), and patients gave informed consent. All supporting data are available within the article or from the corresponding author on reasonable request.

Human atherosclerotic plaques obtained during carotid endarterectomy surgeries at the Department of Vascular and Endovascular Surgery (Klinikum rechts der Isar, Technical University of Munich), as previously described,[3] were co-cultured with patient-matching CD8+ T cells, which were fluorescently stained using a multi-factor labeling strategy with cytoplasmic cell-tracker dyes and DNA markers. After 24 hours of incubation, optical tissue clearing combined with 3D confocal microscopy was performed to examine the precise localization of exogenous CD8+ T cells. Notably, we found that infiltrated exogenous CD8+ T cells were primarily located in areas rich of intraplaque neovessels. In contrast, their presence was rare in the lumen area or at the border area between plaque and tunica media (Figure [A–C]; B, P=0.018 and 0.020, respectively). Interestingly, the majority of exogenous CD8+ T cells were found within close vicinity (<50 µm) of the intraplaque neovessels (Figure [D]) and were also detected inside intraplaque neovessels (Figure [E]). Together, these findings suggest that intraplaque neovessels may facilitate the accumulation of CD8+ T cells.

To gain a deeper insight into the features of the intraplaque neovessels, we reanalyzed the human atherosclerosis single-cell RNA sequencing (scRNA-Seq) dataset from the Munich Vascular Biobank[3] and identified 2 distinct clusters of endothelial cells (EC), designated as *EC1* and *EC2* (Figure [F]). While EC1 expressed several gene markers associated with arterial ECs, EC2 displayed typical venular EC (vEC) gene markers

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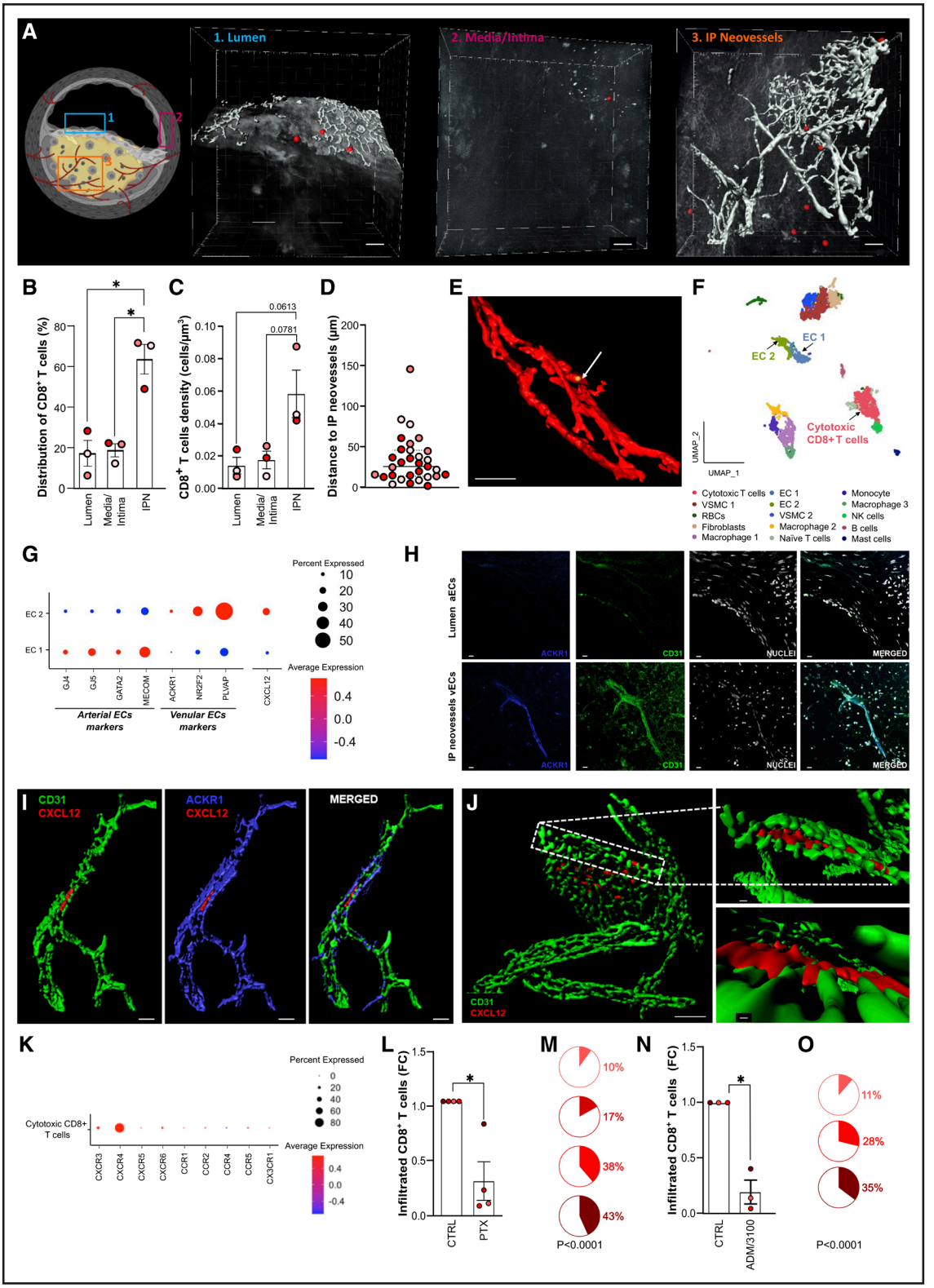

Figure. ACKR1+ neovessels in atherosclerotic plaques recruit CD8+ T cells via CXCL12.
A through **E,** Localization of exogenous CD8+ T cells within human atherosclerotic plaques. Human atherosclerotic plaques were placed on a wetted gelatin sponge raft (Spongostan standard, MS002) at the medium–air interface, and double-labeled exogenous CD8+ T cells (CellTracker Deep Red and DNA marker SYTO 41) were added to the culture medium. After 24 hours, the cultured plaque was fixed (2% paraformaldehyde) and immunostained for PECAM-1 (CD31; clone JC70A). Subsequently tissue clearing (Rapiclear 1.55) was performed followed by 3D confocal microscopy analysis (Leica SP8 coupled to a 16X0.6 immersion objective). **A**, Different regions of the human atherosclerotic plaques were visualized (schematic overview) and representative isosurface renderings (Imaris 8.4) depict the presence of exogenous CD8+ T cells (*red dots*) in (1) lumen, (2) the border between tunica media and intima, and (3) area rich in IPN. IPNs (*grey*) and exogenous CD8+ T cells (*red*) (*Continued*)

Figure Continued. in the microscope datasets were segmented with the Imaris Surface function using the signal derived from CD31 for neovessels and CellTracker Deep Red for CD8+ T cells, the latter of which combined with the DNA marker SYTO 41, which was segmented using the Imaris Spots function. Scale bar, 50 µm. **B**, Distribution of exogenous CD8+ T cells among the assessed locations expressed as percentage of the total number of cells detected in the plaque. Each color represents a different patient (also in **C** and **D**). **C**, Cell density of exogenous CD8+ T cells in the assessed areas. Absolute number of exogenous CD8+ T cells infiltrating various locations of human atherosclerotic plaques was corrected for the volume recorded for each analyzed area per plaque. **D**, Distance between exogenous CD8+ T cells and intraplaque neovessels. The distance from each CD8+ T cell to the closest vascular structure was calculated in Imaris (Distance Transform Matlab Xtension) and then subtracted the mean radius for each cell type. **E**, Representative confocal 3D reconstruction showing an exogenous CD8+ T cell (*yellow dot* and arrow) located within IP CD31+ neovessels (*red*). Scale bar, 50 µm. **F**, UMAP visualization of reclustering of the scRNA-Seq of human atherosclerotic plaques from the Munich Vascular Biobank dataset showing 15 cell populations. Population identities were determined based on marker gene expression. The dataset is available from Gene Expression Omnibus (accession No. GSE247238).[3] **G**, Dot plot visualization showing the expression of arterial marker genes (GJ4, GJ5, GATA2, MECOM), venular marker genes (ACKR1, NR2F2, PLVAP), and CXCL12 in EC1 and EC2, as identified from the scRNA-Seq data (**F**). **H**, Immunohistochemistry staining of 5-µm thick sections of a human carotid plaque showing the expression of ACKR1 (clone 2C3; *blue*) by ECs of IPNs but not by lumen aECs. Nuclei were stained with Hoechst 33342 (*grey*) and ECs with CD31 (*green*). Scale bar: 10 µm. **I**, Isosurface rendering of 3D confocal microscopy data of whole mount, optically cleared human atherosclerotic plaques immunostained with CD31 (NB100-2284; *green*), ACKR1 (*blue*) and CXCL12 (clone 79018; *red*) reveals the presence of CXCL12 in intraplaque ACKR1+ neovessels. Scale bar, 20 µm. **J**, A close-up 3D isosurface rendering obtained with multiphoton microscopy showing that CXCL12 (*red*) is detectable on vECs of IPNs (CD31, NB100-2284; *green*). Scale bar, 30 µm (main rendering) and 10 µm (close-up renderings). **K**, Dot plot visualization showing the expression of chemokine receptors in cytotoxic CD8+ T cells present in human atherosclerotic plaques from scRNA-Seq (**F**). Only chemokine receptors with detectable expression values are shown in this plot. **L** through **O**, Effect of CXCR4 blockade on CD8+ T cells infiltration in human atherosclerotic plaques. Exogenous CD8+ T cells treated with vehicle control (CTRL), labeled in green with Cell Tracker Green, along with those treated with either PTX (20 nM) or AMD-3100 (1 µM), labeled in red with Cell Tracker DeepRed, were added to the culture medium (described in **A** through **E**). After 24 hours, plaques were digested and the presence of exogenous CD8+ T cells was assessed by flow cytometry analysis. **L**, The number of CTRL- and PTX-treated exogenous CD8+ T cells infiltrated in the plaques was determined. Fractions of the initial input of exogenous T cells was calculated, normalized to the CTRL-treated group for each experiment and presented as fold-change relative to CTRL. Each color-coded dot represents a different patient. **M**, Distribution of infiltrated CTRL and PTX-treated exogenous CD8+ T cells in the plaques. The percentage of CTRL (*white*) and PTX-treated (*red*) exogenous CD8+ T cells was calculated for each patient as the proportion of total cells (CTRL+PTX) that infiltrated the plaques. Each color-coded pie chart represents a different patient. **N** and **O**, The same as **L** and **M**, but AMD-3100 was used instead of PTX. Values are shown as mean±SEM. Data were tested for normality using the Shapiro–Wilk test. Statistical significance was determined as follows: repeated measures one-way ANOVA test, followed by Dunnett multiple comparison test (**B**, **C**); 2-tailed, paired 1-sample *t*-test (**L**, **N**); and binomial test followed by Wilson–Brown method to calculate the confidence interval (**M**, **O**). *P* value <0.05 was deemed as statistically significant. ACKR1 indicates atypical chemokine receptor 1; aEC, arterial endothelial cell; CCR1–5, chemokine receptor 1–5; CD8, 31, cluster of differentiation 8, 31; CTRL, control; CXCR1, 3–6, CXC chemokine receptor types 1, 3–6; CXCL12, CXC motif chemokine ligand 12; EC, endothelial cell; FC, fold-change; GATA2, GATA-binding factor 2; GJ4, Gap junction protein alpha 4; GJ5, Gap junction protein alpha 5; IP, intraplaque; IPN, intraplaque neovessels; MECOM, MDS1 and EVI1 complex locus; NR2F2, nuclear receptor subfamily 2, group F, member 2; NK, natural killer cells; PECAM-1, platelet endothelial cell adhesion molecule 1; PLVAP, plasmalemma vesicle–associated protein; PTX, pertussis toxin; RBCs, red blood cells; scRNA-Seq, single-cell RNA sequencing; UMAP, Uniform Manifold Approximation and Projection for Dimension Reduction; vEC, venular endothelial cell; and VSMC, vascular smooth muscle cell.

Nonstandard Abbreviations and Acronyms

ACKR1	atypical chemokine receptor 1
CD8	cluster of differentiation 8
CD31	cluster of differentiation 31
CXCL12	CXC motif chemokine ligand 12
CXCR4	CXC chemokine receptor type 4
EC	endothelial cell
PECAM1	platelet endothelial cell adhesion molecule-1
scRNA-Seq	single-cell RNA sequencing
vEC	venular endothelial cell

(Figure [G]). Consequently, we classified EC1 as luminal arterial ECs and EC2 as vECs of intraplaque neovessels. This classification was confirmed by immunohistochemistry, which showed that ECs lining intraplaque neovessels positively stained for PECAM1 (pan-endothelial cell marker CD31) and the venular marker ACKR1 ([atypical chemokine receptor 1] CD31+, ACKR1+; Figure [H]). In contrast, ECs lining the carotid artery lumen showed positive staining for CD31 but were negative for ACKR1 (CD31+, ACKR1-; Figure [H]).

Because exogenous CD8+ T cells were mainly located in close proximity to and within intraplaque neovessels (Figure [A–E]), we hypothesized that chemokines produced in vEC intraplaque neovessels may facilitate cell migration into human plaques. We specifically focused on CXCL12 (CXC motif chemokine receptor 12), a key causal mediator of coronary artery disease and atherosclerosis.[4,5] Assessment of the expression of CXCL12 in ECs in the Munich Vascular Biobank scRNA-Seq dataset revealed that CXCL12 was highly expressed in the vEC population (Figure [G]). Additionally, 3D multiphoton microscopy of optically cleared human plaques confirmed the presence of CXCL12 in intraplaque neovessels (Figure [I]) and showed that CXCL12 covered the vECs that form intraplaque neovessels (Figure [J]). Additional immunohistochemistry experiments (not shown) demonstrated that the CXCL12 signal was not caused by blood remnants or microthrombi. Together,

the data suggest that CXCL12 is expressed by vECs of intraplaque neovessels.

Next, we assessed whether CXCL12 was involved in the recruitment of CD8+ T cells to human atherosclerotic plaques. First, scRNA-Seq analysis confirmed that CD8+ T cells in human atherosclerotic plaques expressed high levels of CXCR4 (CXC chemokine receptor 4), the conventional chemokine receptor for CXCL12 (Figure [K]). Subsequently, we examined the impact of blocking CXCR4 on exogenous CD8+ T cells recruitment to human plaques. We used pertussis toxin, a broad chemokine receptor inhibitor, and AMD-3100, a US Food and Drug Administration–approved CXCR4 antagonist, and analyzed the recruitment of exogenous CD8+ T cells via flow cytometry analysis. The results indicated that the treatment with both pertussis toxin and AMD-3100 significantly reduced the migration of exogenous CD8+ T cells to atherosclerotic plaques (Figure [L–O]; L, $P=0.025$; M, $P\leq0.0001$; N, $P=0.017$; O, $P\leq0.0001$). Remarkably, the number of AMD-3100–treated exogenous CD8+ T cells in the plaques was 3-fold lower when compared to the vehicle-treated, patient-matching control (Figure [L]), suggesting that the CXCL12–CXCR4 axis serves as a central mediator in the recruitment of CD8+ T cells to human atherosclerotic plaques.

Overall, in using a 3D human atherosclerotic plaque culture model we found that CD8+ T cells expressing CXCR4 are recruited to atherosclerotic plaques via CXCL12 produced by venular intraplaque neovessels. These findings suggest that CXCL12–CXCR4 axis drives CD8+ T cell plaque infiltration and thus, may play a pivotal role in human atherosclerosis.

ARTICLE INFORMATION

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Disclosures

None.

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